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Old yellow enzyme confers resistance of *Hansenula polymorpha* towards allyl alcohol

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Abstract In the methylotrophic yeast, *Hansenula polymorpha*, peroxisomes are formed during growth on methanol as sole carbon and energy source and contain the key enzymes for its metabolism, one of the major enzymes being alcohol oxidase (AO). Upon a shift of these cells to glucose-containing medium, peroxisomes become redundant for growth and are rapidly degraded via a highly selective process designated macropexophagy. *H. polymorpha pdd* mutants are disturbed in macropexophagy and hence retain high levels of peroxisomal AO activity upon induction of this process. To enable efficient isolation of *PDD* genes via functional complementation, we make use of the fact that AO can convert allyl alcohol into the highly toxic compound acrolein. When allyl alcohol is added to cells under conditions that induce macropexophagy, *pdd* mutants die, whereas complemented *pdd* mutants and wild-type cells survive. Besides isolating bona fide *PDD* genes, we occasionally obtained *pdd* transformants that retained high levels of AO activity although their allyl alcohol sensitive phenotype was suppressed. These invariably contained extra copies of a gene cluster encoding homologues of *Saccharomyces carlsbergensis* old yellow enzyme. Our data suggest that the proteins encoded by these genes detoxify acrolein by converting it into less harmful components.

Keywords Detoxification · *HYE* gene cluster · Methylotrophic yeast · NADPH dehydrogenase · Selective peroxisome degradation

Introduction

When *Hansenula polymorpha* cells are grown in media containing methanol as sole carbon and energy source, peroxisomes are formed that contain the key enzymes involved in methanol metabolism, alcohol oxidase (AO), dihydroxyacetone synthase and catalase (van der Klei et al. 1991; Veenhuis and Harder 1991). However, when methanol-grown cells are shifted to conditions of excess glucose, peroxisomes are rapidly degraded by a highly selective process designated macropexophagy (reviewed by Bellu and Kiel 2002; Abeliovitch and Klionsky 2001). Mutants defective in macropexophagy (*pdd* mutants) have been isolated by chemical mutagenesis (Titorenko et al. 1995). The most straightforward technique to isolate the genes affected in these mutants is by functional complementation of the *Pdd* phenotype. Usually, transformants are screened using a colorimetric plate assay based on visualisation of AO activity that remains 6 h after induction of macropexophagy. In this assay, 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) is used as a substrate (Titorenko et al. 1995).

pdd mutant cells do not degrade peroxisomes and hence retain high AO activities while, in functionally complemented strains, most of the peroxisomal AO has been degraded in the vacuole in the 6-h time course. Therefore, complemented strains do not stain significantly in these plate assays, whereas *pdd* mutants produce a blue-green colour. This assay has allowed the isolation of many mutants with a *Pdd* phenotype. However, the assay is laborious, and does not allow positive selection of complemented *pdd* mutants.

This prompted us to find a method that allows positive selection of functionally complemented *pdd* mutants. The method is based on an earlier observation that wild-type *H. polymorpha* cells that contain high levels of AO activity cannot grow in the presence of allyl alcohol (AA; Sibyrny et al. 1989). AA is a substrate for AO, which catalyses its conversion into

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acrolein. This highly reactive substance is toxic for cells and therefore the cells die. We argued that this method could potentially discriminate between cells that are impaired in macropexophagy (and thus maintain high AO activity) and wild-type or complemented *pdd* cells that are susceptible to peroxisome degradation (and thus degrade AO protein). At present, this screen is used for the isolation of putative *PDD* genes.

However, in addition to isolating actual *PDD* genes, we consistently isolated plasmids that suppressed the growth defect of *pdd* cells on plates containing AA but did not complement the actual *Pdd* phenotype. These plasmids contained a gene cluster encoding three homologues of *Saccharomyces carlsbergensis* old yellow enzyme (OYE), a putative NADPH dehydrogenase (Saito et al. 1991). The details of this work are the content of this paper.

Material and methods

Strains and media

All *H. polymorpha* strains used in this study are derivatives of *H. polymorpha* NCYC495 *leu1.1* (Gleeson and Sudbery 1988). *H. polymorpha pdd* mutants were generated either by chemical mutagenesis (Titorenko et al. 1995) or RALF mutagenesis (van Dijk et al. 2001; Komduur et al. 2002). *H. polymorpha* transformants were grown on plates containing 0.67% yeast nitrogen base without amino acids (Difco) and 1.5% granulated agar supplemented with either 1% glucose (YND plates), 0.5% methanol (YNM plates) or 0.4% glucose, together with various concentrations of AA (AA selection plates).

Escherichia coli DH5 α (Sambrook et al. 1989) was used for plasmid constructions and was cultured on LB medium supplemented with the appropriate antibiotics.

DNA procedures

Standard DNA techniques were carried out essentially according to Sambrook et al. (1989). Transformation of *H. polymorpha* was performed as described by Faber et al. (1994). DNA sequencing reactions were performed at BaseClear (Leiden, The Netherlands), using a LiCor automated DNA-sequencer and dye primer chemistry (LiCor, Lincoln, Neb.). For DNA and amino acid sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, USA) was used. The TBLASTN algorithm (Altschul et al. 1997) was used to screen databases at the National Centre for Biotechnology Information (Bethesda, Md.). Protein sequences were aligned using the CLUSTAL X program (Thompson et al. 1997). The nucleotide sequence of the region comprising *HYE1*, *HYE2* and *HYE3* was deposited at GenBank and was assigned accession no. AF486188.

AA and AO plate assays

Growth on plates that contained AA was determined as follows. *H. polymorpha* transformants were replica-plated from YND plates to YNM plates. After 2 days of incubation, colonies were replica-plated to AA selection plates. Growth of colonies was scored 3 days after the transfer. The AO activity plate assay (Titorenko et al. 1995) was used to select functionally complemented *H. polymorpha pdd* transformants.

Results

Positive selection of *H. polymorpha PDD* genes

When *H. polymorpha* cells, pregrown on methanol, are incubated in medium supplemented with AA, peroxisomal AO enzyme activity converts this compound into the highly reactive acrolein, which kills the cells (Izard and Libermann 1978). The use of AA to detect the presence of enzymatically active AO protein was described before, for the isolation of mutants disturbed in AO activity (Sibyryny et al. 1989) and peroxisome biogenesis (Johnson et al. 1999).

We used a variant of this assay to screen large numbers of transformants of *H. polymorpha pdd* mutants for functional complementation. When *pdd* transformants are replica-plated from methanol-containing (YNM) plates to fresh plates containing glucose and AA (AA selection plates), non-complemented colonies die because of the remaining AO activity. Wild-type *H. polymorpha* colonies and complemented *pdd* mutants may survive since, in these strains, the majority of AO enzyme is degraded, along with the peroxisomes. This is illustrated in Fig. 1. Wild-type colonies containing vector pYT3 and $\Delta pdd7$ colonies (Komduur et al. 2002) containing a plasmid with the complementing *PDD7* gene (pYT3-PDD7), show good growth on AA selection plates. In contrast, $\Delta pdd7$ colonies containing the vector pYT3 fail to grow at the same conditions.

We successfully used this method to isolate *H. polymorpha PDD* genes. One example is the *PDD4* gene that we isolated by functional complementation of the *pdd4-43* mutant (Titorenko et al. 1995). The *pdd4-43* mutant

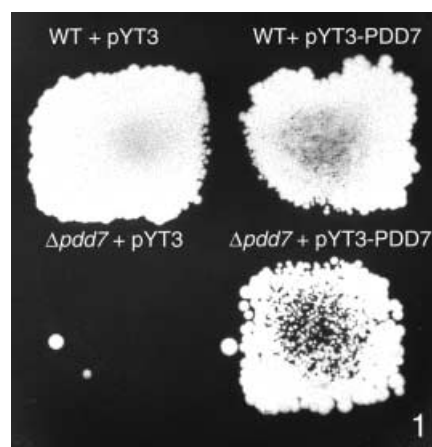


Fig. 1. Allyl alcohol (AA) selection plate assay. Wild-type (*WT*) *Hansenula polymorpha* colonies and $\Delta pdd7$ colonies (Komduur et al. 2002), containing either a plasmid with the entire *PDD7* gene (pYT3-PDD7) or vector pYT3 were pre-grown on methanol-containing plates and replica-plated to AA selection plates containing 0.08 mM AA. After 3 days of incubation at 37 °C, the wild-type colonies and the complemented $\Delta pdd7$ colony show good growth, whereas the uncomplemented $\Delta pdd7$ colony fails to grow at the same conditions

was transformed using a *H. polymorpha* gene library in vector pYT3 (Tan et al. 1995). Some 5,000 leucine-prototrophic transformants were tested on AA selection plates containing 0.08 mM AA, and three colonies appeared to grow after 3 days of incubation at 37 °C. Subsequently, these transformants were analysed using the colorimetric AO plate assay to screen for colonies restored in peroxisome degradation (Titorenko et al. 1995). Indeed, one of the transformants showed true complementation of the Pdd phenotype of the *pdd4-43* mutant. The other transformants did not display typical macropexophagy characteristics, as exemplified by the retained high levels of AO activity (data not shown). Currently, we are analysing the role of the *PDD4* gene in selective peroxisome degradation in detail. This indicates that the AA selection method in principle provides an easy screening method to isolate *PDD* genes by functional complementation.

Overexpression of genes encoding homologues of OYE allows growth of *H. polymorpha* *pdd* mutants on AA plates

As described above, we observed that the AA selection method also resulted in the isolation of plasmids that suppressed the growth defect of *pdd* mutants on AA plates, whilst not complementing their Pdd phenotype. We observed this phenomenon infrequently in multiple independent transformation experiments using various *pdd* strains (data not shown). Analysis of this type of plasmid isolated from mutants *pdd9-F52* and *pdd11-M* indicated that they contained either identical or overlapping genomic *H. polymorpha* inserts. To understand more as to why these plasmids suppress the growth defect of *pdd* cells on AA selection plates, we decided to analyse the gene(s) causing this phenotype. Two plasmids containing overlapping fragments were selected (Fig. 2). Subcloning revealed that plasmid pHYE-2.5, containing an approximately 2.5-kb *Bam*HI genomic fragment, allowed slow growth of *pdd9-F52* colonies on

plates containing 0.1 mM AA, as compared with transformants that contained the original plasmid, pHYE-9 (Fig. 2). This suggested that not the entire DNA region responsible for the suppression phenotype was present on pHYE-2.5. Initial sequencing of this fragment demonstrated that it contained part of a gene cluster of highly similar genes (Fig. 2). Subsequently, we isolated the entire gene cluster that was located on an approximately 4.7-kb genomic region. Partial sequencing upstream and downstream from this region revealed no additional genes belonging to the same family (data not shown). Analysis of the sequence showed that the genomic region contained three highly similar genes (Fig. 2). They encoded proteins with high similarity to OYE, a putative NADPH dehydrogenase from brewer's bottom yeast (*S. carlsbergensis*; Saito et al. 1991). An alignment of *S. carlsbergensis* OYE and the *H. polymorpha* homologues is presented in Fig. 3; and similarities between the *H. polymorpha* gene products and with a series of relevant homologues are shown in Table 1. Consistent with the nomenclature used in *Kluyveromyces lactis* (*KYE1*; Miranda et al. 1995), we designated the *H. polymorpha* genes *HYE1*, *HYE2* and *HYE3* (Fig. 2; see GenBank accession number AF486188).

Analysis of the primary sequences of *HYE1*, *HYE2* and *HYE3* showed that, in these proteins, most residues comprising the co-factor flavin mononucleotide (FMN)-binding site as determined for *S. carlsbergensis* OYE are conserved (Thr38, Gly73, Gln115, Arg244, Gly325, Asn326, Phe327, Gly346, Gly348, Arg349 in OYE; Karplus et al. 1995). Of the putative active site residues (His192, Asn195, Tyr197, Tyr376 of OYE; Fig. 3; see Karplus et al. 1995), only the two tyrosine residues are conserved in all *HYE* enzymes. In contrast, in *HYE2* and *HYE3*, Asn195 is not conserved, but is replaced by histidine; and both His192 and Asn195 are converted into non-conserved residues in *HYE1* (Fig. 3). The effect of these changes on the activity of the *HYE* enzymes is unknown.

Overproduction of OYE in both wild-type *H. polymorpha* and *pdd* mutants allows growth at enhanced concentrations of AA

To investigate whether (over-) expression of the plasmid-based *H. polymorpha* *HYE* gene cluster resulted in a general increase in the ability of *H. polymorpha* colonies to grow on AA selection plates, we transformed *H. polymorpha* wild-type, *pdd2-1*, *pdd5-J40* and $\Delta pdd7$ cells with plasmids containing the entire *HYE* gene cluster (pHYE20; see Fig. 2), using the empty vector pYT3 as control. After selection of transformants on YND plates, colonies were replica-plated to YNM plates, grown for 2 days at 37 °C and finally replica-plated to AA selection plates containing 0.08 mM and 0.16 mM AA. Growth was scored 3 days after replica-plating. The results (Table 2) indicate that the plasmid containing the entire *HYE* gene cluster allowed both

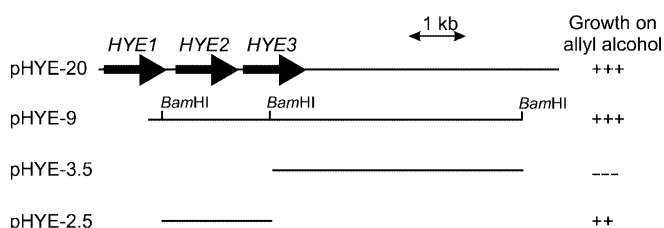


Fig. 2. Schematic representation of the genomic region comprising the *H. polymorpha* *HYE* gene cluster. Indicated are the two genomic regions of the plasmids pHYE-9 and pHYE-20 that suppressed the growth defect of *H. polymorpha* *pdd9-F52* and *pdd11-M* cells respectively, in the presence of AA. In addition, two subclones, pHYE2.5 and pHYE3.5, are shown. The ability of the plasmids to suppress the growth defect of *pdd9-F52* in the presence of AA is indicated to the right. +++ Good growth after 3 days of incubation at 37 °C, ++ weaker growth, --- no growth. Only relevant restriction sites are indicated

Fig. 3. Sequence alignment of the deduced primary sequences of *H. polymorpha* HYE1, HYE2 and HYE3 with that of *S. carlsbergensis* old yellow enzyme (OYE; PIR-A39495), using the one-letter code. Gaps were introduced to maximise the similarity. Identical and conserved residues are shaded. Active site residues in OYE are indicated by an arrow

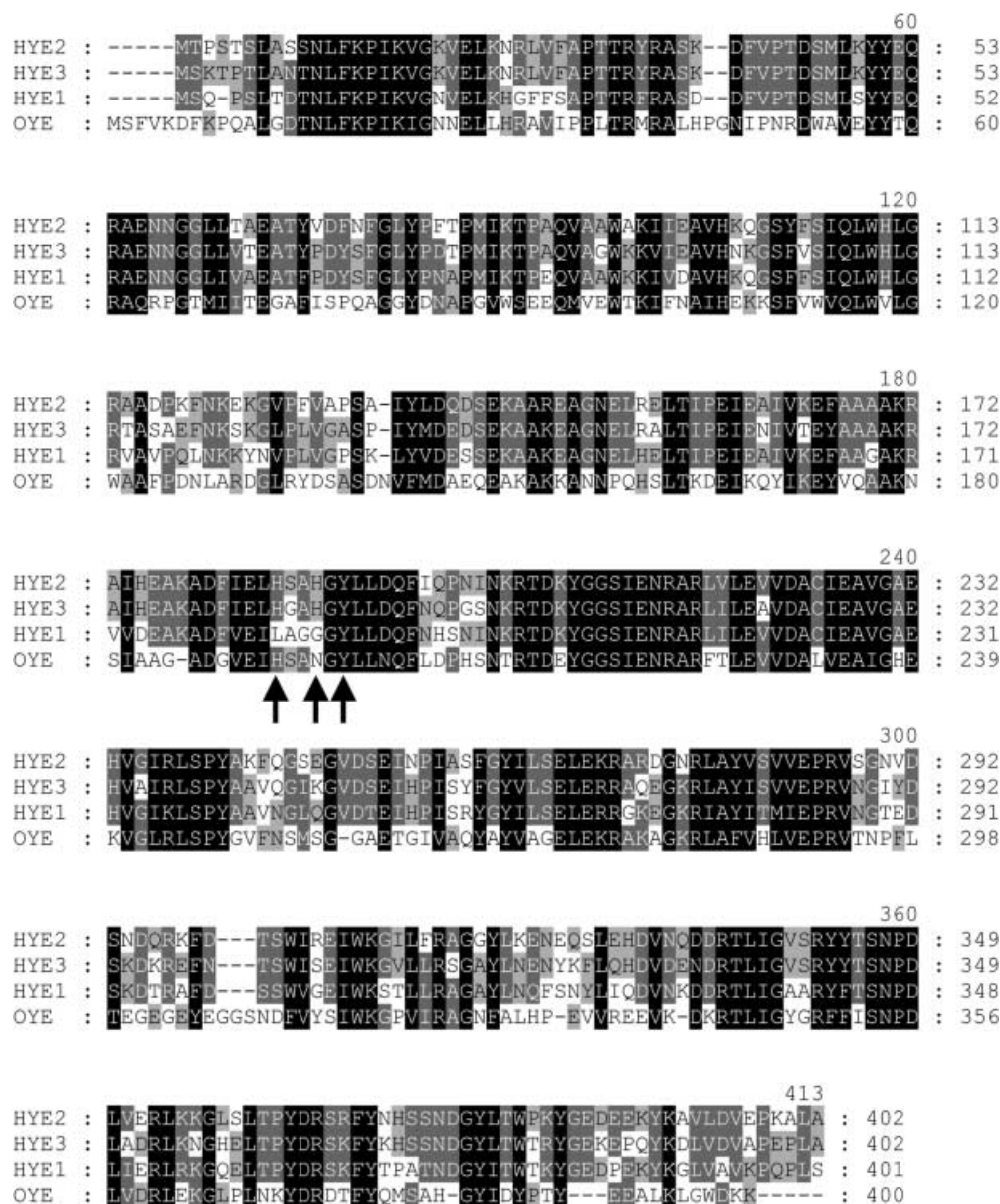


Table 1. Sequence identities between *Hansenula polymorpha* HYE1, HYE2 and HYE3 and their counterparts in other organisms. Protein sequences of *H. polymorpha* HYE1, HYE2 and HYE3 were aligned with putative orthologues of other eukaryotes: *Saccharomyces carlsbergensis* old yellow enzyme (OYE; PIR-A39495), *S. cerevisiae* OYE2 (PIR-A46009) and OYE3

(PIR-A55569), *Kluyveromyces lactis* KYE1 (PIR-S55844), *Candida albicans* oestrogen-binding protein (EBP; PIR-A36990), *Arabidopsis thaliana* 12-oxophytodienoate reductase (OPDA; EMBL-CAA71627) and *Pseudomonas putida* morphinone reductase (MorB; PIR-S64687). Sequence identities (%) between the proteins are indicated.

	HYE1	HYE2	HYE3	OYE	OYE2	OYE3	KYE1	EBP	OPDA	MorB
HYE1	100									
HYE2	69	100								
HYE3	70	76	100							
OYE	41	42	41	100						
OYE2	42	43	42	92	100					
OYE3	41	41	40	80	82	100				
KYE1	39	41	42	72	72	68	100			
EBP	46	47	50	44	45	44	43	100		
OPDA	33	34	32	37	37	40	34	32	100	
MorB	33	34	37	36	37	39	34	36	41	100

Table 2. Effect of the expression of the plasmid-based *HYE* gene cluster in *H. polymorpha* on growth on allyl alcohol (AA) selection plates. Colonies were grown on methanol-containing media and, after 2 days, shifted to AA selection plates. Growth was scored after 3 days of incubation at 37 °C. + Growth, – no significant growth. Overexpression of the *HYE* gene cluster allows growth on enhanced concentrations of AA in both *pdd* mutants and wild-type *H. polymorpha*

Strain	Allyl alcohol (mM)	
	0.08	0.16
Wild type (pHYE-20)	+	+
Wild type (pYT3)	+	–
$\Delta pdd7$ (pHYE-20)	+	+
$\Delta pdd7$ (pYT3)	–	–
<i>pdd2-1</i> (pHYE-20)	+	+
<i>pdd2-1</i> (pYT3)	–	–
<i>pdd5-J40</i> (pHYE-20)	+	–
<i>pdd5-J40</i> (pYT3)	–	–

mutant and wild-type *H. polymorpha* colonies to grow on plates containing 0.16 mM AA, a concentration at which wild-type controls containing pYT3 are unable to grow. This indicates that the *HYE* gene cluster promotes growth of *H. polymorpha* colonies in the presence of AA.

Discussion

Positive selection of *PDD* genes by the AA screening method

Here, we describe a positive selection method for the isolation of genes that are involved in selective peroxisome degradation (macropexophagy) in the methylotrophic yeast *H. polymorpha*. The method is based on the ability of *H. polymorpha* cells not affected in macropexophagy to grow on enhanced concentrations of AA after induction of peroxisome degradation. These conditions are lethal for *pdd* cells, due to the formation of acrolein from AA by the high AO activity that remains in these cells. The AA assay has been used successfully in our group to isolate several *PDD* genes via functional complementation.

We observed that, in addition to *PDD* genes, the described method also selects for genes that suppress the growth defect of *pdd* mutants on AA selection plates, but do not complement the *Pdd* phenotype of these mutants. We have identified these genes as homologues of OYE which, in *H. polymorpha*, is encoded by at least three genes present in a gene cluster, which we designated *HYE1*, *HYE2* and *HYE3*.

The presence of the *HYE* gene cluster as a set of genes suppressing the growth defect of *pdd* mutants on AA selection plates may interfere with the isolation of actual *PDD* genes. This potential drawback is overcome by using the AA assay as an initial screen, followed by an analysis of the few colonies that survive the AA selection method by a subsequent AO activity plate assay. Therefore, suppression by the *HYE* gene

cluster may not represent a major disadvantage of our selection method.

It must be noted that, besides screening for complemented *pdd* mutants, the described method also allows isolation of novel *pdd* mutants. Using the recently described RALF gene-tagging technique (van Dijk et al. 2001), we obtained approximately 1,500 transformants and identified 50 colonies that were unable to grow on plates containing 0.08 mM AA after induction of macropexophagy. At least seven of these showed a strongly reduced ability to degrade peroxisomes, as judged from reduced AO degradation patterns (our unpublished data). Thus, the AA method is a powerful tool that can be used to isolate *PDD* genes by functional complementation and also to identify novel *H. polymorpha pdd* mutants.

Overproduction of OYE probably suppresses the growth defect on AA by detoxification of acrolein

OYE was first isolated almost 70 years ago from brewer's bottom yeast (*S. carlsbergensis*; Warburg and Christian 1933). It was the first enzyme found to require a cofactor (FMN) for its function (reviewed by Karplus et al. 1995; Schopfer and Massey 1991). OYE has been described to exhibit NADPH oxidoreductase activity using oxygen as terminal electron acceptor. OYE can also reduce olefinic bonds of α , β -unsaturated ketones and aldehydes via its co-factor FMN (Vaz et al. 1995). Although the physiological function of OYE in yeast is still unknown; functions have been attributed to OYE homologues in other organisms. For instance, in *Pseudomonas putida*, the homologous enzyme was shown to be an active morphinone reductase, an enzyme involved in the degradation of morphine (French and Bruce 1995). Furthermore, the *Arabidopsis thaliana* enzyme was shown to contain 12-oxophytodienate (OPDA) reductase activity, which is involved in signalling in plant-defensive reactions (Schaller and Weiler 1997). In the latter study, it was also demonstrated that yeast OYE could reduce OPDA. Our data indicate that over-expression of the *HYE* gene cluster in *H. polymorpha* from a plasmid confers enhanced resistance towards higher concentrations of AA. In *H. polymorpha*, AA is converted by AO into the toxic compound acrolein, which, like morphinone and OPDA, is an unsaturated aldehyde/ketone. We hypothesise that, in *H. polymorpha*, the *HYE* gene cluster encodes enzymes that can also reduce α , β -unsaturated aldehydes and therefore may detoxify acrolein by converting it into propionaldehyde, which is less harmful to cells (Slott and Hales 1985). In line with this, the ability of wild-type *H. polymorpha* cells to grow on plates containing 0.08 mM AA may reflect the net result of a balance between the endogenous detoxifying activity of the *HYE* gene products and the residual AO activity after induction of macropexophagy. In this manner, the acrolein that is formed under these conditions is effectively

detoxified by HYE activities. In contrast, *pdd* mutants retain high AO activity, which promotes acrolein formation to toxic levels. However, artificially enhanced HYE protein levels allow both *pdd* mutants and wild-type *H. polymorpha* to grow on higher concentrations of AA.

In conclusion, our data suggest that the HYE proteins in *H. polymorpha* and possibly OYE-like proteins in general, have a function in the detoxification of dangerous α , β -unsaturated ketones and aldehydes. In fact, other NADPH dehydrogenases (e.g. thioredoxin reductase, glutathione reductase) are known to have roles in the detoxification of reactive oxygen species (Costa and Moradas-Ferreira 2001; Moradas-Ferreira et al. 1996).

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